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Phlebotomine sandflies of Botswana: a taxonomic review and a faunistic update with the first record of genus *Phlebotomus*

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ABSTRACT

The first records of phlebotomine sandflies from Botswana have been published only recently, comprising of four species of genus *Sergentomyia*. This update presents the first record of genus *Phlebotomus*, namely *Ph.* (*Anaphlebotomus*) *rodhaini* Parrot, which is also the first detection of a putative vector of leishmaniasis in Botswana. In addition, records of the *Sergentomyia* "*bedfordi* (Newstead) group" are reviewed, and the molecular taxonomy of all taxa known from Botswana is analysed based on three mitochondrial gene fragments (mtDNA). The presence of *Se. congolensis* (Bequaert and Walrveus) and *Se. salisburiensis* (Abonnenc) is confirmed, whereas the previously mentioned *Se. caliginosa* Davidson and unassigned specimens of the "*bedfordi* group" are proposed to belong to the tentatively named *Se. bedfordi* "Maun" form. The mtDNA analyses confirmed the species delimitations. For the first time, portions of the *ND5* gene were used for the purpose of sandfly molecular taxonomy. This gene revealed a high inter-specific variability and may thus be applied as an alternative molecular marker for future studies.

1. Introduction

Sandflies (Psychodidae: Phlebotominae) are haematophagous nematoceran Diptera that are, in contrast to mosquitoes, blackflies, and biting midges, independent from aquatic environments, as their offspring develops strictly terrestrial, e.g. in animal burrows and shelters, rock cracks, or caves. In many regions of the world they are obligate vectors of kinetoplastid parasites of the genus *Leishmania*, the causative agents of leishmaniasis infection. Major human disease foci are found across Africa (WHO, 2010), with the exception of southern Africa, where there are only small and scattered foci known in Namibia (Noden and van der Colf, 2013) and Zambia (Alvar et al., 2012).

The knowledge on the Afrotropical sandfly fauna increased between 1951 and 1993 from 58 to 147 species and subspecies (Kirk and Lewis, 1951; Seccombe et al., 1993). The zoogeographical southern African sub-region with the Rhodesian Highland, the Southeast Veldt and the Southwest Arid districts (Kirk and Lewis, 1951), covering Namibia, Botswana, Zimbabwe, South Africa, Lesotho, Swaziland, as well as parts of Angola, Zambia, and Mozambique harbours about 51 species, 32 of which are endemic to southern Africa (Seccombe et al., 1993). Recently the first records of phlebotomines from Botswana were reported (Krüger, 2015, 2016), namely *Sergentomyia* (*Grassomyia*) *inermis* (Theodor, 1938), *Se.* (*Ser.*) *congolensis* (Bequaert and Walravens, 1930), *Se.* (*Ser.*) *caliginosa* Davidson, 1987, and *Se.* (*Ser.*) *salisburiensis* (Abonnenc, 1967), the latter three of which belong to the *Se.* "*bedfordi* (Newstead, 1914) group". If based on small sample sizes, misinterpretations of morphological characters in these small-bodied and closely related species can easily happen due to damaged body parts or suboptimal preparation of the microscopic slides containing the structures of interest such as cibarial armature, pharynx, and genitalia. Analyses of further samples, and comparison with the initial specimens revealed the presence of two additional taxa one of which replaces the erroneously recorded *Se. caliginosa*.

Taking advantage of the availability of specimens of closely related species from South Africa, Namibia, and Cameroon, data on the molecular taxonomy of Botswana sandflies were also retrieved by conducting DNA sequence comparisons of the mitochondrial genes for *cytochrome b* (*cytB*) and *cytochrome c oxidase I* (*COI*). The latter is the most commonly used region for DNA barcoding (Hebert et al., 2003) and has previously been analysed in Indian *Sergentomyia* (Pradeep Kumar et al., 2012), whereby *cytB* analyses have already been conducted on sandflies from Afghanistan and elsewhere (Krüger et al., 2011). In addition, the 5′ portion of the mitochondrial gene for the *NADH dehydrogenase* subunit 5 (*ND5*)

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was analysed for the first time in order to evaluate its suitability for phlebotomine taxonomy (Ye et al., 2015).

2. Material and Methods

Details of the study area, sampling data, trapping technique, morphological sample preparation, and species identification of 28 specimens have been published recently (Krüger, 2015). In brief, the study area was in the vicinity of Maun, northern Botswana, and the centre of Ngamiland district. The region was very flat at an elevation of about 940 m above the sea level, and characterised by the unique combination of an inland river delta, surrounded by savannah and semi-desert. The sampling device was a Standard CDC light-trap (Model 512; John W. Hock, Gainesville, FL), deployed 50 cm beside a termite hill at a fan height of 50 cm, about 10–200 m off Thamalakane river. One collection site was in Sexaxa ward, in the premises of the Okavango Research Institute (ORI), the second site in a private property in Disaning ward. The trap was run from 5 pm to 8.30 am during seven trap nights.

An additional nine specimens (five males and four females), whose analysis is reported here, have the same details and were processed accordingly (Table 1).

Each mounted specimen was documented photographically and identified using the morphological keys provided by Abonnenc (1972) and Davidson (1990), the latter from which the taxonomy and terminology used here for the *Sergentomyia* "*bedfordi* group" was adopted.

For genomic DNA extraction, ethanol-preserved, unmounted body parts (thorax and proximal parts of the abdomen, legs, wings) of each sandfly of interest were dried for 3 h at 37 °C, and subsequently processed with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following Qiagen's supplementary protocol for purification of total DNA from insects, using disposable microtube pestles for homogenisation. For comparison, further specimens of closely related species from Afghanistan (Kondoz town, collected 2009; Krüger et al., 2011), Cameroon (Mokolo town, collected 2012, unpublished) and South Africa (KwaZulu Natal, collected 2015 by A. Latif, unpublished) were processed, i.e. *Se. distincta* (Theodor, 1933), *Se. schwetzi* (Adler et al., 1929), *Se.* (*Gra.*) *dreyfussi* ssp. *turkestanica* Theodor & Mesghali, 1964, *Se.* (*Gra.*) *ghesquierei* (Parrot, 1929), and *Se.* (*Gra.*) *squamipleuris* (Newstead, 1912).

All PCRs were performed in a Biometra TProfessional Basis thermocycler (Biometra, Göttingen, Germany). The protocols for *cytB* and *COI* PCRs were described as previously (Ready et al., 1997; Krüger et al., 2011; Pradeep Kumar et al., 2012), using GoTaq G2 Hot Start and GoTaq G2 Flexi polymerase, respectively (Promega, Mannheim, Germany). The only modification was for *cytB* reactions a 40 s annealing during the first five cycles (instead of only 30 s; Ready et al., 1997). For *ND5* amplification, an approximately 510 base pair long fragment at the 5′ end of the *ND5* coding region was chosen, as this was shown to be the most variable region of the entire sandfly mitochondrial genome (Ye et al., 2015). The following primers were used: forward N5-J-6579 (modified from Simon et al., 1994) TTCWCTYCAWCCTTGATC; reverse N5-6500 (modified from Birungi and Munstermann, 2002) GCBGGYTTTTATTCWAAGGA. PCR amplifications were performed in 50 μl volumes containing 1 μl of DNA template, 9 μl Go Taq Flexi buffer (Promega, Mannheim, Germany), 3 μl 25 mM $MgCl_2$, 0.2 pmol/μl of each primer, 0.3 μl 10 mM dNTP mix, and 1.5 U of Go Taq Hot Start polymerase. The thermal cycler conditions were the same as for *cytB* PCR (see above).

All reactions were run at 2.2% FlashGel cassette agarose gels (Lonza, Rockland, USA). Positive reactions were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and commercially sequenced by direct forward and reverse sequencing (Seqlab, Göttingen, Germany). The respective sequences were deposited in Genbank under accession numbers KY451779-KY451798 (*COI*), KY451799-KY451811 (*ND5*), KY451812-KY451833 (*cytB*).

The *cytB* (422 base pairs) and *ND5* (491 bp) sequences were individually run against the BLAST tool of the publicly available NCBI Genbank in order to search for highly similar sequences. Further, they were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and analysed in MEGA7 (Kumar et al., 2016). Neighbour-Joining (NJ) analyses were computed using the Kimura 2-Parameter method. Bootstrap values were calculated to test the robustness of the resulting taxon ID trees and were obtained by conducting 1000 replicates.

For *COI* barcoding, the obtained sequences (533 bp) were initially run against two databases, i) by using the BLAST tool, and ii) the specimen identification tool of BOLD4 (Barcode of Life Data systems v4 (http://v4.boldsystems.org/index.php). In order to visualise the barcoding results, *COI* sequences were aligned and analysed as described above, in this case together with Genbank entry JX105042 for *Ph. rodhaini* Parrot, 1930 and BOLD reference sequence BNITM095-12 for *Se.* (*Gra.*) *dreyfussi* ssp. *turkestanica* from Afghanistan (detailed specimen records and sequence information including traces files can be found within the BOLD Working Group 1.4 Initiative "Human Pathogens and Zoonoses": "Ticks and Sandflies at BNITM Hamburg 2012").

3. Results

3.1. Morphotaxonomy

In addition to the 28 previously investigated specimens, another nine were morphologically identified to species level. The new specimens belonged to five species, three of which had been reported previously (Table 1): *Se.* (*Gra.*) *inermis* (two additional specimens), *Se.* (*Ser.*) *congolensis* (one more specimen) and *Se.* (*Ser.*) *salisburiensis* (two additional specimens). One specimen that was previously assigned to *Se.* (*Ser.*) *caliginosa* of the "*bedfordi* group", six specimens that were previously treated as "*bedfordi* group" sensu lato, and three new specimens, turned out to belong to a new taxon tentatively named *Se. bedfordi* "Maun" form. A single female fly was unequivocally identified as *Phlebotomus* (*Anaphlebotomus*) *rodhaini*.

Three of the recorded species belong to the *Se.* "*bedfordi* group" and are rather delicate to distinguish. Updated morphometric measurements are summarised in Table 2. For comparison, data for *Se. distincta*, as well as published data for other relevant species (see below) are listed, i.e. for *Se. caliginosa*, *Se. formica* Davidson, 1987 and *Se. bedfordi* s.str.

The decision for (re-)assigning 10 specimens to the new "Maun" form of *Se. bedfordi* was based on the observation that both males and females showed character combinations close to or intermediate between *Se. caliginosa* and *Se. formica*.

In females of the "Maun" form, the shape of the cibarial pigment plate resembles that of *Se. caliginosa* and, to a lesser degree, of *Se. bedfordi* s.str., being oblong with a variable median projection (Fig. 1). In addition, the posterior margins are strikingly ragged. However, intermediate or *Formica*-like characters are as follows: cibarial armature with 41-50 closely packed, long fine, monomorphic teeth [according to Davidson (1990) 45–65, versus 35–42 for *Se. caliginosa*; see Table 2]; median posterior margin of hard palate with tuberculate denticles, though small (smooth in *Se. caliginosa*); apical margin of hypopharynx with deep indentations (weakly undulating in *Se. caliginosa*).

Males of the "Maun" form ($n = 3$) of *Se. bedfordi* exhibited the following characters: cibarial armature with 16–23 small, irregular teeth (16–24 in *Se. caliginosa*, versus 30–42 in *Se. formica*); pos-